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FOREWORD

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INTRODUCTION AND BACKGROUND

Our objective is to obtain a useful blood substitute with normal adult HbA as the starting material. In order for such a derivative to be useful it should have a low oxygen affinity so that oxygen can be readily released to the tissues. The experimental procedures should be as uncomplicated as possible and the materials should not be unduly expensive. To this end we have been studying the carboxymethylation of the N-terminal amino groups of HbA. The initial studies on this derivative showed that it had a low oxygen affinity, a desirable property for a blood substitute (1). An earlier annual report under this contract described the preparation of this derivative by a relatively simple procedure.

The second objective of the studies is to cross-link this carboxymethylated Hb so that it will be retained longer in the circulation and yet maintain a significant degree of Hb function. For this purpose we are investigating the use of several types of crosslinking agents in order to obtain a stable cross-linked Hb of molecular weight 128,000, i.e. two cross-linked tetramers with a low oxygen affinity. The starting material that we chose is the well-characterized 64,000 cross-linked Hb, DBBF-Hb, that we obtain from Dr. John Hess's laboratory in Rockville, MD. This material has the desired low oxygen affinity and is highly purified and well-characterized.

We also decided to find a simple procedure to determine Hb subunit dissociation by gel filtration. The purpose was to accurately distinguish the amount of 128,000 material from the amount of 64,000 material and also to determine whether the dimeric $\alpha\beta$ pairs, as well as the individual α and β chains, were unaffected by the second chemical crosslinker. Therefore, the focus of this report will be on this procedure that has just been completed. It will be summarized in this report.

Amino acid replacements or cross-links at various locations on the hemoglobin molecule may influence its physical-chemical and functional properties. For example, the substitution of Glu-6(β) \rightarrow Val (Ingram, 1956) on the exterior of sickle hemoglobin (HbS) has profound effects on the solubility of the protein and consequently on its impaired oxygen delivery to tissues that ultimately leads to a severe clinical situation. Amino acid replacements at the $\alpha_1\beta_2$ subunit

interface, an important region that undergoes structural shifts during the transition between the oxy and deoxy tetrameric states (Perutz, 1989), can also affect hemoglobin function. Since the dissociation behavior of the tetramer will affect its oxygen binding properties, such information for any natural or recombinant or crosslinked hemoglobin is important in order to interpret the effects of the amino acid substitution(s) on function. Dissociation of tetrameric hemoglobin is also a very important issue in blood substitute research since dimers are rapidly cleared from the circulation (Bunn and Jandl, 1968). Knowledge of the sites and nature of the amino acid replacements that influence dissociation is also important in understanding the dynamics of the $\alpha_1\beta_2$ interface. A rapid, sensitive method is required to determine how replacements at different locations on the hemoglobin tetramer or chemical cross-links affect the degree of dissociation at the $\alpha_1\beta_2$ interface to form dimers. We chose sickle Hb to verify the validity of the method since an accurate tetramer-dimer K_d has been reported for it by an independent method.

RESULTS

Gel Filtration of Dissociable Hemoglobins - Hemoglobin either in the O_2 - or the CO-form was applied to a Superose-12 HR 10/30 column on a Pharmacia FPLC system and eluted with 150 mM Tris Ac buffer, pH 7.5 at a flow rate of 0.4 ml/min. Different concentrations of hemoglobin, each in 100 μ l, were applied and the absorbance of the eluent was measured at 405 nm with the Pharmacia on-line mercury lamp detection system with a 5 mm flow cell. With the 100 μ l sample load, the dilution factor during elution measured by the width at half-height (ml) of the peak divided by the sample load volume, was found to be 6.1 ± 0.4 for HbA. This value was constant over the hemoglobin range used to determine the tetramer-dimer dissociation constant. As will be discussed later, much of this dilution is due to mixing both before and after passage through the column and not to dilution during gel filtration on Superose-12. There was no dissociation of heme from the globin nor oxidation to met Hb since the spectrum of the eluted hemoglobin was identical to that of the original sample. The absorbance of the

dimer was assumed to be the same as an equivalent weight of the tetramer. Amino acid analysis after acid hydrolysis of the eluted protein gave the correct amino acid composition of hemoglobin.

The elution times were highly reproducible both for different amounts of the tetrameric and dimeric species (described below), as well as for the same concentration of each dissociable Hb. Purified and well-characterized crosslinked hemoglobin A tetramers, DBBF-Hb (Vandegriff et al., 1989; Chatterjee et al., 1986), which is currently under study in blood substitute research, or DIBS-Hb (Manning et al., 1991), were used to determine the elution position of undissociated tetrameric Hb; several concentrations ranging from 8.0 nM to 1.8 μ M eluted at the same peak position, $13.28 \pm .04$ ml (3 determinations) (Fig. 1, peak 1). The peak elution position of the $\alpha\beta$ dimer was determined with the natural dimeric mutant Hb Rothschild, (Trp-37(β) \rightarrow Arg), which is completely dissociated to dimers in the oxy conformation (Gacon et al., 1977; Sharma et al., 1990). Concentrations ranging from 4.3 μ M to 34.5 μ M eluted at the same position, $14.28 \pm .05$ ml (3 determinations) (Fig. 1, peak 3).

Dissociation of Natural and Recombinant Sickle Hemoglobins - Dissociable hemoglobins eluted as a single peak whose position varied between tetrameric and dimeric hemoglobins (Fig. 1, peak 2) when the concentration was in the range of the K_d value. With higher concentrations, the peak position of dissociable hemoglobins was closer to that of the tetrameric Hb, whereas at lower Hb concentrations, it was closer to the dimer position. All elution profiles had comparable shapes representing a mixture of dimeric and tetrameric hemoglobin in rapid equilibrium. The percentage of tetramer present can be readily estimated from the elution position of the peak (V) relative to the elution volumes for the dimer (V_d) and tetramer (V_t) (Eq. 3 below). Sickle hemoglobin was chosen to evaluate the reliability of the K_d values obtained by this procedure since association constants for natural HbS reported by Williams and Kim (1976) were obtained by a completely independent method, i.e. ultracentrifugal analysis. A plot of the percent Hb tetramer as a function of hemoglobin concentration gave a hyperbolic-like curve (Fig. 2), which closely resembled the profile described by Tiselius (1942) for a

general equilibrium process during column chromatography, by Andrews (1964), by Ackers and Thompson (1965) and by Chiancone et al. (1968) for hemoglobin. The figure inset shows a linear transformation of this data whose abscissa intercept yields the tetramer-dimer dissociation constant $K_d=0.42 \mu\text{M}$. Since Hb tetramer-dimer K_d values are increased in the presence of chloride and at lower pH values (Chu and Ackers, 1981), the value we find for HbS is in general agreement with the value $K_d=2.5 \mu\text{M}$ reported by Williams and Kim (1976) at pH 7.0.

Recombinant sickle hemoglobin expressed, purified, and characterized as described previously (Martin de Llano et al., 1993, 1994), was found to have a K_d value of $0.75 \mu\text{M}$ at pH 7.5 (Fig. 3); this value is about the same as that for natural HbS within experimental error. These results are consistent with our earlier conclusions that hemoglobin expressed in the yeast system is identical to the corresponding natural hemoglobin purified from erythrocytes and forms the basis for the studies on the other recombinant hemoglobins described below.

Dissociation of Recombinant Sickle Hb Double Mutants - The aggregation of sickle Hb in the deoxy conformation is initiated by the interaction of the external Val-6(β) substitution with a hydrophobic site comprising Leu-88(β) on the exterior of an adjacent tetramer (Martin de Llano et al., 1993a,b and refs. therein). In order to ascertain the strength of this primary site of tetramer aggregation, we have reported its substitution by Ala resulted in a significant increase (30%) in the concentration needed for gelation, i.e. aggregation was impeded (Martin de Llano et al., 1994). Although it is the deoxy HbS tetramers that aggregate in the erythrocyte, any increased dissociation of oxy Hb would affect the overall equilibrium to give a decreased concentration of deoxy HbS tetramers and hence a reduced extent of aggregation. Since it was conceivable that the amino acid replacement of Ala for Leu-88(β) affected its structure in the oxy conformation in such a way that there was an increased tetramer-dimer dissociation, the K_d of this recombinant HbS L88A(β) double mutant was determined. It was found to be $0.56 \mu\text{M}$ (Fig. 4), which is of the same order of magnitude as the values for natural and recombinant HbS described above. These results show that this amino acid replacement in the double mutant has little effect on the dissociation behavior of the $\alpha_1\beta_2$ interface in the oxy conformation of

hemoglobin. Thus, the effect of the amino acid substitution in the recombinant Hb on the aggregation process is a direct one at the level of the tetrameric interaction.

Recombinant Hemoglobins with Substitutions in the $\alpha 1\beta 2$ Subunit Interface - Two recombinant hemoglobins, D99K(β) and N102A(β), with substitutions in the $\alpha 1\beta 2$ interface at Asp-99(β) and Asn-102(β) respectively, were expressed in order to study their roles in the oxy \rightarrow deoxy transition (Yanase et al., 1994,1995). Using the method described above, the oxygenated form of the N102A(β) mutant was found to be about 400 times more dissociated than HbA, like the natural Hb Kansas with a Thr at position 102 of the β -chain (Bonaventura and Riggs, 1968).

For the D99K(β) recombinant Hb, there was no concentration-dependence of the peak elution position as a function of hemoglobin concentrations ranging from 0.05 to 4.2 μ M. At each concentration the peak elution was in the position of the $\alpha\beta$ dimer with no indication of any movement into the position of the tetramer. We have reported that this particular recombinant Hb in the oxy conformation gives two bands upon isoelectric focusing (Yanase et al., 1994). The mass of the α and β chains, as well as their heme moieties, were found to be identical. However, the possibility remained that one of the components was an $\alpha\beta$ dimer and the other an $\alpha_2\beta_2$ tetramer that for some reason had different pI values. In order to test this possibility, each component was eluted from the IEF gel and then subjected to the FPLC gel filtration procedure described above. Each was found to elute in the position of dimeric Hb, thus excluding the above possibility.

The natural hemoglobin mutant, Hb Yakima, was chosen for study because its substitution is at the same site as that for the D99K(β) recombinant Hb, but with a His rather than a Lys. Its K_d value, 0.10 μ M (Fig. 5), indicates that it is tetrameric and indeed less dissociated than HbA, in agreement with the value of 0.06 μ M reported by Benesch and Kwong (1995) and 0.09 μ M found by Turner et al. (1992) for this natural Hb mutant. Thus, Hb Yakima displays properties that bear little similarity to those of the D99K(β) mutant. Even

though both substitutions contain side chains that bear some positive charge, it is apparently their relative size that causes the extreme disparity in their dissociation behavior.

Tetramer-Dimer Dissociation of Carboxymethylated Hemoglobin - Chemically-modified hemoglobin with the covalently bound anion, $-\text{CH}_2\text{COO}^-$, at the N-terminus of each of its four chains has been reported previously to have a low oxygen affinity since the carboxymethylated group mimicks the bound CO_2 group at the same position (Di Donato et al., 1983). The tetramer-dimer dissociation constant of this chemically-modified hemoglobin was calculated to be $1\ \mu\text{M}$ (Fig. 6). This value is consistent with that reported by Benesch and Kwong (1995) and by Turner et al. (1992) using different procedures.

Effect of Allosteric Regulators on the Tetramer-Dimer Dissociation of Ligand Hemoglobin - Even though it is recognized that allosteric regulators of hemoglobin exert their effects by binding to tetrameric Hb in the deoxy conformation, there have been some reports that the oxy conformation has a binding site for such regulators. The procedure described above can readily resolve such a question. When HbA in the range of its K_d value was applied to Superose-12 either in the absence or presence of DPG or IHP, there was not an increased elution into the tetramer range. If allosteric regulators had bound to the tetrameric state, the tetramer-dimer equilibrium would have been expected to shift into the tetramer range.

DISCUSSION

There are several existing procedures to measure tetramer-dimer dissociation constants of hemoglobins. For example, Edelstein (1966), Kellett and Schachman (1971) and Williams and Kim (1976) described the use of the ultracentrifuge to measure tetramer dissociation. Benesch and Kwong (1990, 1995) and Hargrove et al. (1994) reported a technique based on transfer of heme from hemoglobin dimers to albumin (Bunn and Jandl, 1968) to measure subunit dissociation. Other procedures, such as dialysis techniques (Guidotti et al., 1963) have also been used. Using gel filtration on Sephadex, Andrews (1964), Ackers and Thompson (1965), Chiancone et al. (1968), Baudin-Chich et al. (1988) and Fronticelli et al., (1994) have reported

dissociation constants of various hemoglobins. These investigators overcame the problem associated with peak broadening on Sephadex columns by analyzing the leading and trailing edges of the elution zones. The newer Superose-12 gel filtration medium used in the studies described below does not lead to inordinate broadening since discrete, reproducible peaks that eluted in less than 2 ml were obtained. The time for each analysis is short so that a dissociation constant for a particular Hb can be obtained in approximately a day with a high degree of accuracy. The K_d values we found for several natural and a chemically modified hemoglobin are in good agreement with reported values.

Zimmerman and Ackers (1971) carried out two elegant simulation experiments for gel filtration on Sephadex. In one they assumed identical amounts of protein with varying assumed dissociation constants. The calculated positions of the peaks on the column did not appear to correlate with these assumed dissociation constants. Dilution of the protein on the column may have been a factor since if one calculates the protein concentrations, they are comparable to those that they show. In the second simulation experiment they assumed a dissociation constant and varied the protein concentration applied to the column. The apparent dissociation constant, calculated from the "elution volumes" was five orders of magnitude less than the value assumed in the simulation. However, if one assumes that the elution volumes and dissociation constant are valid, one can calculate that the protein dilution on the column was about 50-fold. Some small degree of dilution may occur on the column in our experiments so that the K_d values reported here could be somewhat underestimated. However, they are certainly well within an order of magnitude of the values reported using different techniques on some of the same hemoglobins.

MATERIALS AND METHODS

Hemoglobins - Natural HbS was purified from the red cells of sickle cell anemia patients (Martin de Llano, 1993a,b). The recombinant hemoglobins - HbS and the double sickle mutant (L88A(β) with Leu-88(β), part of the receptor site for Val-6(β), replaced by Ala

(L88A(β)) were expressed and purified as described previously (Martin de Llano, 1994, 1995). Two recombinant hemoglobins with substitutions in the $\alpha_1\beta_2$ interface, Asp-99(β) \rightarrow Lys (D99K(β)) and Asn-102(β) \rightarrow Ala (N102A(β)) were also expressed and purified as described previously (Yanase et al., 1994, 1995). The chemically modified hemoglobin (Cm-Hb), with carboxymethyl groups at the N-terminals of both α - and β -chains, was prepared by reductive alkylation and purified as described previously (Di Donato et al., 1983). The naturally-occurring hemoglobin Yakima (Asp-99(β) \rightarrow His) and Hb Rothschild (Trp-37(β) \rightarrow Arg) were generously provided by Dr. Ruth Benesch. Each gave a single band by isoelectric focusing and the molecular weight of the latter was confirmed by mass spectrometry, which was kindly performed by Drs. Urooj Mirze and Brian Chait. The preparation of the cross-linked tetrameric DBBF-HbA with a fumaryl group covalently linked between the two Lys-99(α) side chains in the central dyad axis (Vandegriff et al., 1989; Chatterjee et al., 1986) and DIBS-HbS crosslinked between the N-terminals of the 2 α -chains (Manning et al., 1991) have been fully characterized.

Curve Analysis - Within experimental error the peak widths at half height were constant over the range of Hb concentrations and the peak heights were also found to be directly related to the amount of Hb injected. Thus, a plot of log of detector response vs. the log of the Hb concentration injected gave a straight line with slope near unity (Fig. 7). Hence, the dilution based in the widths at peak half-heights are comparable and do not vary with the extent of dissociation. In the concentration range studied, there was no inadvertent flattening of the elution profiles that could conceivably lead to a change in elution peak position. The elution peaks were reproducibly narrow eluting in less than 2 ml and were somewhat skewed towards the trailing edge. There are two possible origins of this elution peak asymmetry. First, it could be of instrumental origin due mainly to mixing before and after passage through the column and possibly due also to the electrical damping of the detection system. The second possibility, in the case of equilibrating mixtures of tetramer and dimer species, could be due to retardation at the lower protein concentrations on the leading and trailing edges of the peak due to the

relatively greater amount of the dimeric species present at low protein concentrations. We concluded that the observed asymmetry was largely of instrumental origin since it did not change with hemoglobin concentration. Since the peak width and asymmetry did not change with the degree of dissociation, equilibration between dimers and tetramers must be very rapid because there was no indication of broadening to yield separate peaks of dimeric and tetrameric species.

Evaluation of Kd Values - The percentage of tetramer present was calculated from the elution curves as follows:

Assuming that the elution volume varies with Log (Molecular weight) and the molecular weight of the dimeric species is M, it follows that:

$$\text{Log } (2)/(V_d - V_t) = \text{Log } (\text{Mol. Wt.}/M)/(V_d - V) \quad (1)$$

where V_d , V_t and V are the elution times for the dimeric, tetrameric, and a mixture of species, respectively.

The effective molecular weight is the "weight average" molecular weight, which is given by:

$$\begin{aligned} M_w &= \Sigma(N_i \cdot M_i^2) / \Sigma(N_i \cdot M_i) \\ &= M[4(\%T) + 2(100 - \%T)] / [2(\%T) + 2(100 - \%T)] \\ &= M(1 + \%T/100) \end{aligned} \quad (2)$$

where M is the molecular weight of the dimeric species and $\%T$ is the percentage of the tetramer present. Combining equations 1 and 2 gives both:

$$\%T = 100(M_w/M - 1) = 100(2^{(V_d - V)/(V_d - V_t)} - 1) \quad (3)$$

$$\text{and } V = V_d - ((V_d - V_t) (\text{Log}(1 + \%T/100) / \text{Log } 2)) \quad (4)$$

The tetramer dissociation constant K_d was estimated as follows:

If the maximum amount of hemoglobin tetramer is $[H]$ and the concentrations of dimeric and tetrameric species are $[D]$ and $[T]$, respectively, so that $\%T = 100 [T]/[H]$, it follows that:

$$\begin{aligned} K_d &= [D]^2/[T] = 4([H] - [T])^2/[T] \\ &= [(100 - \%T)^2 [H]] / (25 \%T) = 0.04(100 - \%T)^2 [H] / \%T \end{aligned} \quad (5)$$

$$\text{Hence, } \text{Log}(K_d) = \text{Log}[H] - \text{Log} \frac{[\%T]}{0.04 (100-\%T)^2} \quad (6)$$

Thus, a plot of $\text{Log} \frac{\%T}{0.04(100-\%T)^2}$ with respect to $\text{Log}[H]$ will yield a straight line,

of slope 1. When $\frac{\%T}{0.04(100-T)^2} = 1$, $K_d=[H]$

The quadratic equation 5 can also be solved in terms of (%T)

$$\%T = (8.[H] + K_d) - [K_d^2 + 16.K_d.[H]]^{1/2}/(0.08[H]) \quad (7)$$

From Eq. 7 one can conclude that when $[H]=K_d$ then $\%T=61\%$ and hence, from Eq. 4 that $(V_d-V)/(V_d-V_t)=69\%$. These values can be used to obtain reliable estimates for K_d for the non-linear regression analyses. It should be noted that, because the elution volume varies empirically with the log of the effective molecular weight, one does not get 50% tetramer when $(V_d-V)=(V_d-V_t)/2$ but rather, from Eq. 3, when the elution peak position appears midway between the dimer and tetramer peak positions, $\%T=100(\sqrt{2}-1)=41.4\%$.

Equations 4 and 7 were used to evaluate K_d , and the errors in K_d , by non-linear regression analyses of the values of the elution volumes (V) with respect to the hemoglobin concentrations ($[H]$). These non-linear regression analyses were performed with the graFit PC software program which was also used to prepare the figures. It should be noted that one cannot use simple linear regression with data plotted as in the figure insets since the variances are not comparable and, furthermore, the analysis dictates that the data should lie on a line of unit slope (Eq. 6).

Potential Systematic Errors - Since the values calculated for K_d depend on the values for the elution volumes of the dimeric (V_d) and tetrameric (V_t) species, we routinely re-evaluated V_d and V_t with standard tetrameric and dimeric hemoglobins with each set of results. However, if a wide range of hemoglobin concentrations, $[H]$, is used ($4K_d < [H] < K_d/4$), it is possible to evaluate not only K_d , but also V_d and V_t by curve fitting the experimental elution volume data for the unknown. When we analyzed such data in this way, we generally got very good agreement with the values of V_d and V_t determined with standards. For example, the

data in Fig. 2 yielded values of $K_d = 0.35 \pm 0.11 \mu\text{M}$, $V_d = 14.31 \pm 0.04 \text{ ml}$ and $V_t = 13.35 \pm 0.05 \text{ ml}$ compared to the standard dimeric and tetrameric hemoglobins, which gave $V_d = 14.3 \text{ ml}$ and $V_t = 13.3 \text{ ml}$, respectively. However, unless there is a large number of data points, the calculated errors for K_d are much greater if three parameters are allowed to vary than when two of the parameters (V_d and V_t) are determined independently from the standard dimeric Hb Rothschild and tetrameric crosslinked Hb. Furthermore, it is not always possible to use a high enough Hb concentration to convert most of it to the tetrameric form (e.g. the recombinant Hb N102A(β)). The analysis above pertains to the dissociation of the tetramers only to dimers. The good agreement between calculated V_d and V_t values and those obtained with authentic standards provides evidence that the assumption is valid under the experimental conditions described. The absence of monomers is also indicated by the lack of peak broadening under such conditions.

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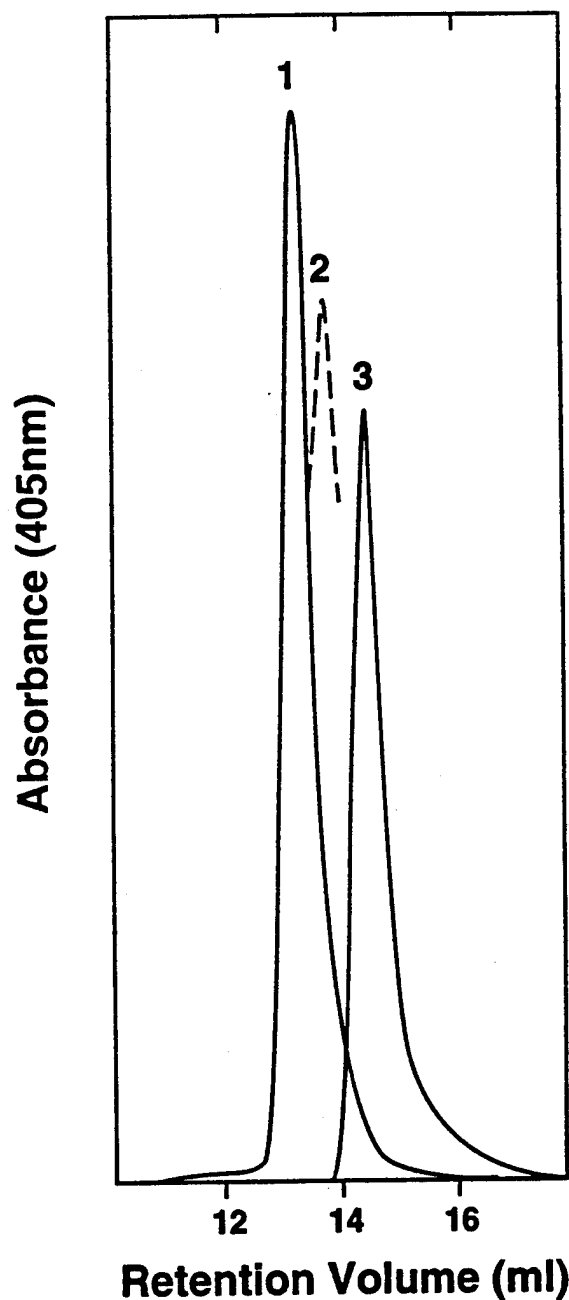


Fig. 1. Elution Profile of Dissociable Hemoglobins - Peak 1 is the elution position of crosslinked tetrameric Hb, DBBF-Hb or DIBS-Hb; peak 2 is the variable position of dissociable hemoglobin tetramers dependent on the hemoglobin concentration; peak 3 is the position of dimeric hemoglobin determined with Hb Rothschild as described in the text. No difference was found for the elution position in 75 mM or 150 mM Tris-Ac, pH 7.5.

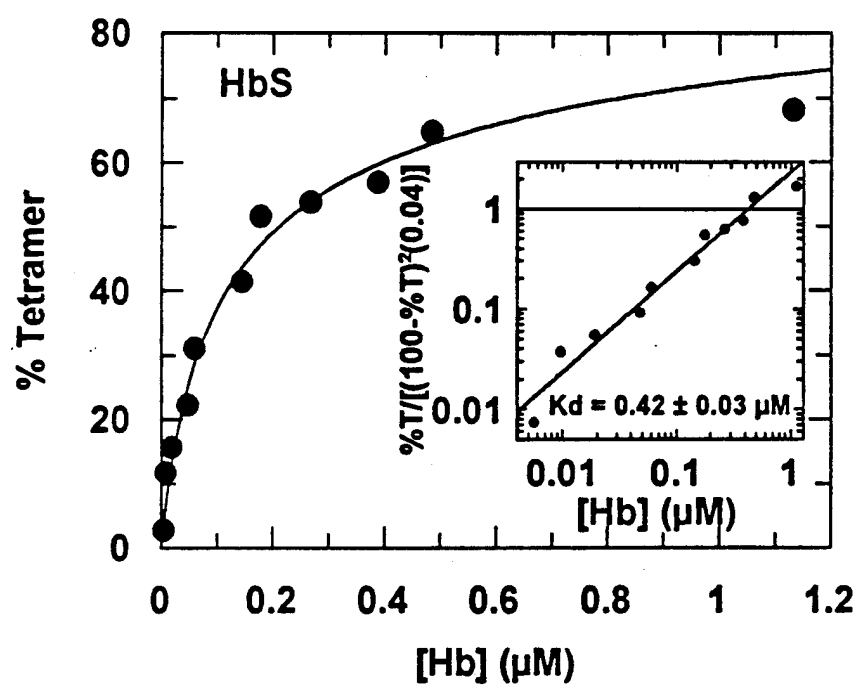
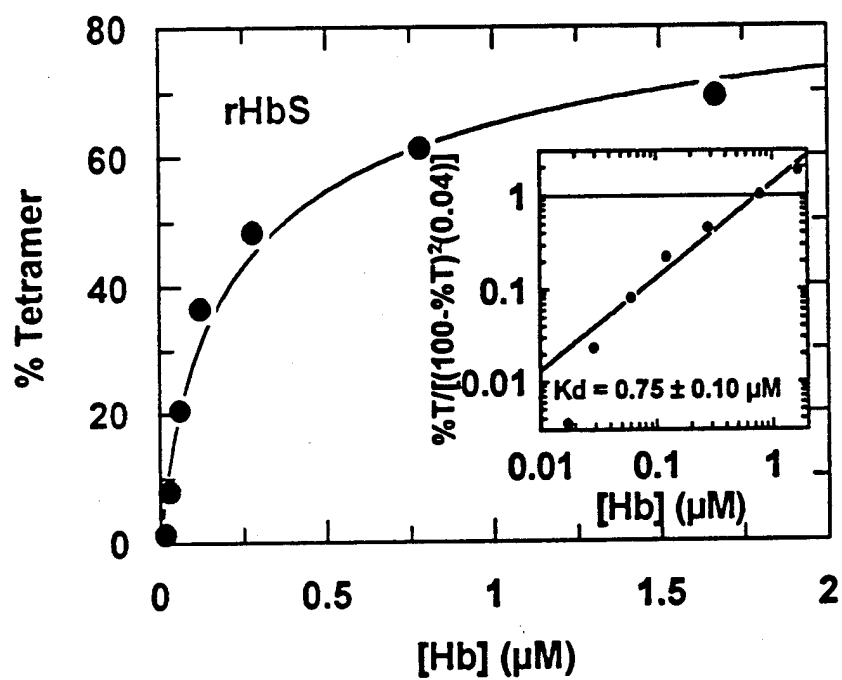


Fig. 2. K_d of natural HbS. The procedure used for calculation of this, as well as the K_d values in the other figures, are described in detail in the text.

Fig. 3. K_d of recombinant HbS.

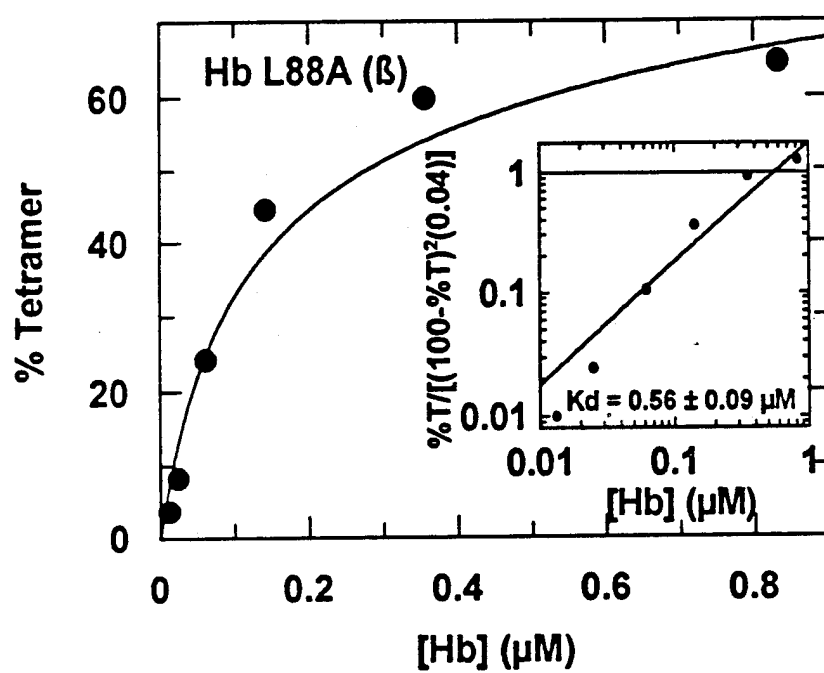


Fig. 4. K_d of recombinant L88A(β) sickle Hb double mutant.

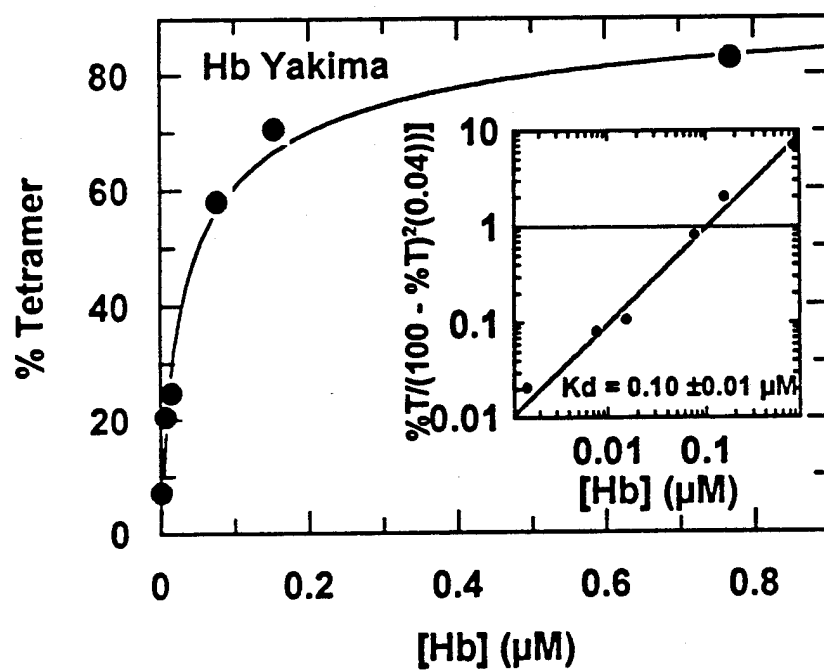


Fig. 5. K_d of natural mutant Hb Yakima.

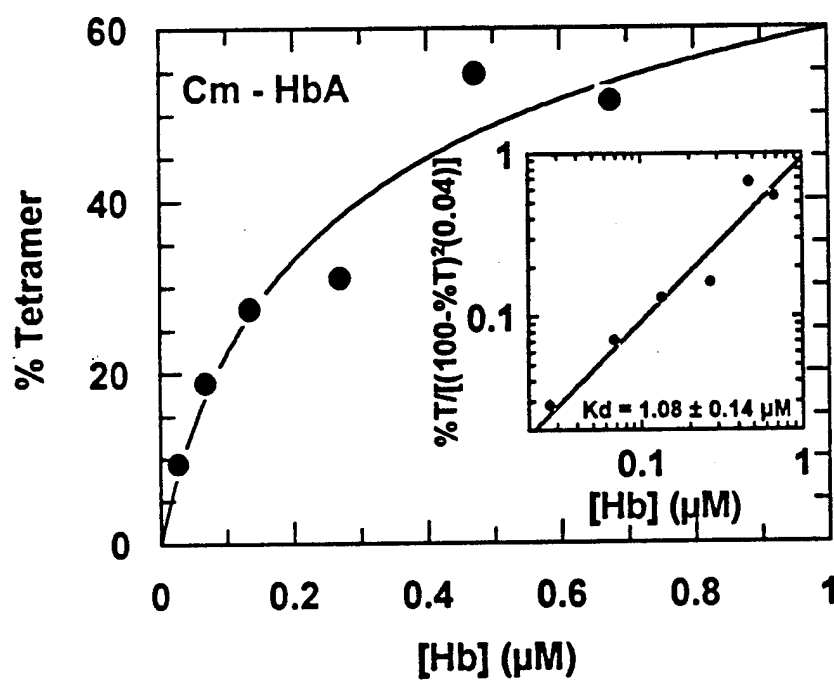


Fig. 6. K_d of chemically modified carboxymethylated hemoglobin.

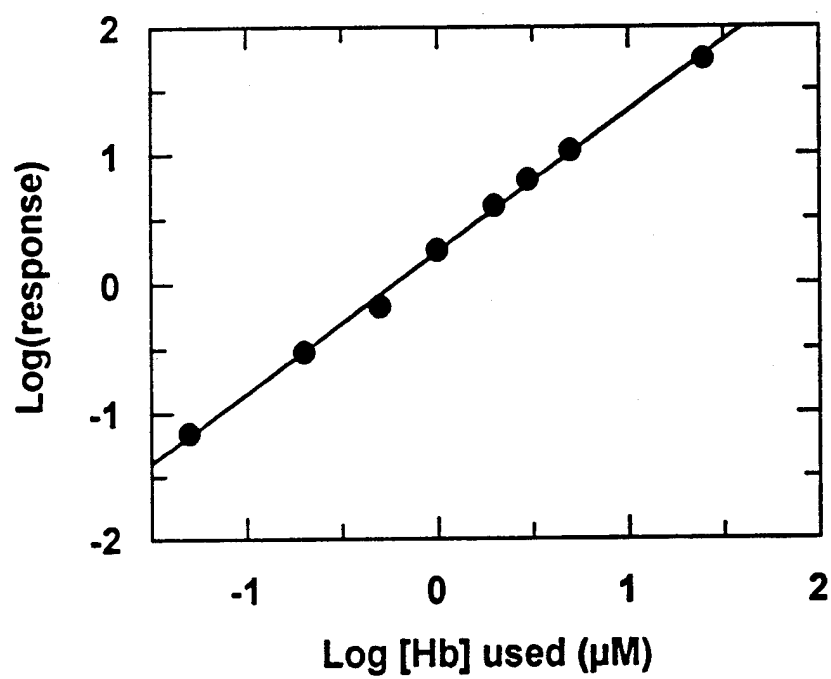


Fig. 7. Detector response as a function of applied hemoglobin concentration.

PUBLICATIONS RESULTING FROM THIS CONTRACT:

- Yanase, H., Cahill, S., Martin de Llano, J.J., Manning, L.R., Schneider, K., Chait, B.T., Vandegriff, K.D., Winslow, R.M., and Manning, J.M. (1994) Properties of a Recombinant Human Hemoglobin with Aspartic Acid 99(β), an Important Inter-Subunit Contact Site, Substituted by Lysine. *Protein Science* 3, 1213-1223.
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GRADUATE DEGREES: None